

Table 1.
Properties Of Some Proteases Associated With Post-Translational Processing

Protease	Subcellular Localization	Tissue Distribution	Cleavage Signal
furin	Golgi	ubiquitous	RXKR (SEQ ID NO:2)
MMP-2	Golgi	tumor cells	PLGLWA (SEQ ID NO:3)
MT1-MMP	plasma membrane	tumor cells	PLGLWA (SEQ ID NO:3)
caspase-1	secretory pathway	ubiquitous	YEVDGW (SEQ ID NO:4)
caspase-2	secretory pathway		VDVADGW (SEQ ID NO:5)
caspase-3	secretory pathway		VDQMDGW (SEQ ID NO:6)
caspase-4	secretory pathway		LEVVDGW (SEQ ID NO:7)
caspase-6	secretory pathway		VQVDGW (SEQ ID NO:8)
caspase-7	secretory pathway		VDQVDGW (SEQ ID NO:9)
alpha-secretase	secretory pathway	ubiquitous	amyloid precursor protein (APP)
proprotein convertase (subtilisin/kexin isozyme SK1-1)	endoplasmic reticulum	ubiquitous	brain neutrophilic growth factor precursor (RGLT) (SEQ ID NO:10)
tumor associated tyrosin		tumor cells	
foot and mouth disease virus, protease 2A			NFDLLKLAGDVESNPGP (SEQ ID NO:11)--

Please replace the paragraph at page 23, line 10 with the following amended paragraph:

--An alternative to relying on cell-associated proteases is to use a sequence encoding a self-cleaving linker. In one embodiment of the invention, the foot and mouth disease virus (FMDV) 2A protease is used as linker. This is a short polypeptide of 17 amino acids that cleaves the polyprotein of FMDV at the 2A/2B junction. The sequence of the FMDV 2A propeptide is NFDLLKLAGDVESNPGP (SEQ ID NO:11). Cleavage occurs at the C-terminus of the peptide at the final glycine-proline amino acid pair and is independent of the presence of other FMDV sequences and cleaves even in the presence of heterologous sequences.--

Please replace the paragraph at page 30, line 8 with the following amended paragraph:

--The H protein cytoplasmic tail comprises the amino-terminal 34 amino acids of the protein (sequence: NH₂- MSPQRDRINAFYKDNPHPKGSRIVINREHLMIDR-COOH) (SEQ ID NO:12). Modification of H protein by removal of the 24 amino acids immediately following the initiator methionine (AA 2-25) results in a loss of fusogenic activity by the virus. In contrast, deletion of either 8 amino acids immediately following the initiator methionine (amino acids 2-9 deleted) or 14 amino acids between amino acids 2 and 17 (amino acids 3-16 deleted) enhance the fusogenic activity of the virus (Cathomen et al., 1998, J. Virol. 72:1224). These results indicate

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that membrane proximal amino acids 17 to 25 comprise a sequence necessary for fusion activity. The results also indicate that amino acids at least between those numbered 2 – 16 are involved in negative regulation of fusogenic activity. Therefore, in one embodiment, fusogenicity is enhanced by deletion of either amino acids 2-9, or 3-16. In a further embodiment of the invention, amino acids 2-16 are deleted. In still a further embodiment of the invention, amino acids 2-24 are deleted, and preferably 2-20. In one embodiment of the invention, the virus comprises a truncated H sequence comprising 8-14 fewer amino acids.--

Please replace the paragraph at page 31, line 2 with the following amended paragraph:

as
--In one embodiment, the F protein is modified by alteration of its cytoplasmic tail, which comprises the carboxy-terminal 33 amino acids of the protein (sequence: NH₂-RGRCNKKGEQVGMSRPGLKPDLTGTSKSYVRSL-COOH) (SEQ ID NO:13). Modifications of the F protein found to increase the fusogenic activity of the virus include addition of unrelated sequences to the C-terminus (for example, by alteration of the normal stop codon, or other means), and deletion of 16 or 24 C-terminal amino acids (Cathomen et al., 1998, J. Virol., supra). Viruses incorporating these changes include faster formation of syncytia, with a concomitant enhancement in the rate of cell killing. In another embodiment of the invention, a modified virus is provided which comprises a C-terminal tail comprising at least one deletion and/or substitution and which has enhanced fusogenic activity and/or cell killing effects. The addition or exchange of sequences have also been demonstrated to enhance the fusogenicity of the virus. Therefore, in a further embodiment of the invention, the F protein is modified by the addition of 28 amino acids, while in still a further embodiment, the C-terminal tail of the F protein is exchanged with the C-terminal tail of a Sendai virus.--

Please replace the paragraph at page 36, line 12 with the following amended paragraph:

as
--The invention thus contemplates the introduction of selected cancer-associated protease cleavage sites into viruses. The cleavage of the F₀ precursor may be made dependent on a protease other than furin by replacement of the furin cleavage signal R-R-H-K-R (SEQ ID NO:14) at amino acids 108 to 112 of the measles virus (MV) or the corresponding residues of the other Paramyxoviridae viruses with that of another protease. Cleavage by furin occurs after

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Amend
arginine 112. Correct cleavage at this site is essential, because changing arginine 112 to leucine has been shown to result in aberrant cleavage and loss of fusion ability (Alkathib et al., 1994, J. Virol. 68:6770).--

Please replace the paragraph at page 37, line 23 with the following amended paragraph:

a7
--The F primer should have sequences complementary to the F₀ template sequence 3' of the nucleotides encoding the furin cleavage sequence (i.e., complementary to F₁ sequence adjacent to the cleavage site), preceded by (i.e., 3' of) a randomized stretch of 12 bases corresponding in position to those encoding the furin cleavage site RHKR (SEQ ID NO:15). In order to avoid restoring furin cleavage, the "randomized" stretch of nucleotides should not be truly random, but rather be designed such that positions 1, 2 and 3 cannot be lysine or arginine (see Example 5).--

Please replace the paragraph at page 46, line 9 with the following amended paragraph:

as
--To produce pCGHFurCP, primers Fur CP1.Nf and SfurinCP were used in a PCR reaction with pro-insulin template (ATCC) for 18 cycles at 94°C 1 min, 55°C 1 min and 72°C for 1 min. The resulting PCR fragment, tagged with Sfi-CP-Not was gel purified and digested with SclI and NotI restriction enzymes. The pCGH backbond pCGH EGFr-was also digested with SfiI and NotI, and ligated with the prepared insert. Ligation reactions were transformed into competent *E. coli*, and resulting colonies screened by PCR for the presence of C-peptide insert. Successful transformants were grown to large scale and the DNA isolated.

FurCP1.Nf

ttt tcc ttt tgc ggc cgc ttt cat caa cgc ttc tgc agg gac ccc tc (SEQ ID NO:16)

SFurinCP

gtc cat gcg gcc cag ccg gcc CGA TTA AAG AGA gag gca gag gac ctg cag gtg gg
(SEQ ID NO:17)

V H A A Q P A R L K R E A E D L Q V (SEQ ID NO:18)--

Please replace the paragraph at page 47, line 1 with the following amended paragraph:

ag --To produce pCGHG4SCP, primers G4SCP1.Nf and SG4SCP.b were used in a PCR reaction as described above.

G4SCP1.Nf

ttt tcc ttt tgc ggc cgc ttt cat cat caa cgc ttc tgc agg gac ccc tc (SEQ ID NO:19)

SG4SCP.b

gtc cat gcg gcc cag ccg gcc GGT GGA GGC GGT TCA gag gca gag gac ctg cag gtg gg

(SEQ ID NO:20)

V H A A Q P A G G G G S E A E D L Q V (SEQ ID NO:21)--

Please replace the paragraph at page 48, line 7 with the following amended paragraph:

210 --F protein cytoplasmic tail mutations have been introduced to plasmids encoding full length measles virus genomic RNA (e.g., p(+)MV, Radecke et al., supra) as follows. Plasmids peFHLP, peFHLF and peFHLI (described in Schmid et al., 1992, Virology 188: 910) and plasmid peF (cSeV)HL were used as starting material for generation of full length measles virus plasmids encoding the following F mutants, respectively: mutant Fc+28, resulting from a stop codon mutation, comprises 28 amino acids of extraneous sequence appended to the C-terminus of the wild-type F protein (Fc+28 also has an additional Glu relative to the Edmonston B strain of the virus at position 27 relative to the transmembrane domain); mutant FcΔ16 lacks the 16 C-terminal amino acids of the F cytoplasmic tail (remaining sequence of the cytoplasmic tail is RGRCNKKGEQaGMSRPG (SEQ ID NO:22), where the lower case "a" also differs from the sequence of the Edmonston B strain of measles virus); mutant FcΔ24 lacks 24 C-terminal amino acids relative to the wild-type virus (remaining sequence of the cytoplasmic tail is RGRCNKKGE (SEQ ID NO:23)); and mutant FcSeV has the measles virus F protein cytoplasmic tail replaced by the F cytoplasmic tail from Sendai virus. Plasmid pcF (cSeV)HL was generated by subcloning a PstI –PacI PCR fragment encoding the SeV F cytoplasmic tail into peFHL. PCR was performed with pGem4-SVG₀ (described in Vidal et al., 1989, J. Virol. 63:

Q10
Q11
892) as the template and primers 5'-AAACTGCAGACTCAAAGGTCAATGC-3' (SEQ ID NO:24) and 5'-CCCTTAATTAATATACAGATCTCAACGGAT-3' (SEQ ID NO:25).--

Please replace the paragraph at page 49, line 10 with the following amended paragraph:

Q11
--H protein mutant plasmids peHcΔ8 (lacking amino-terminal amino acids 2-9), peHcΔ14 (lacking amino terminal acids 3-16), and peHcΔ24 (lacking amino-terminal amino acids 2-25) were constructed by subcloning a ClaI-EcoRI PCR fragment into peH5. peH5 is a shuttle vector for subcloning into the full-length p(+)MV (Cathomen et al., J. Virol., supra). peH5 contains a single ClaI site in the 5' untranslated region of the H protein and a single EcoRI site at the border between the transmembrane domain and the ectodomain, both sites introduced by silent mutations. PCR was performed with peH5 DNA as template and forward primers 5'-CCATCGATAATGGCCTTCTACAAAGATAACC-3' (peHcΔ8) (SEQ ID NO:26), 5'-CCATCGATAATGAGCCATCCCAAGGGAAGTAGG-3' (peHcΔ14) (SEQ ID NO:27), and 5'-CCATCGATAATGAACAGAGAACATCTTATGATT-3' (peHcΔ24) (SEQ ID NO:28). The reverse primer annealed downstream of the H protein coding region in the plasmid.--

Please replace the paragraph at page 49, line 21 with the following amended paragraph.

Q12
--To construct the H mutant in which the measles virus cytoplasmic tail was replaced with the Sendai virus H cytoplasmic tail, fusion PCR was performed. The SeV H-tail encoding region was amplified from the pGem4-SVHN plasmid template (described by Vidal et al., supra) with primers 5'-CCATCGATAATCATGGATGGTGATAGGGG-3' (SEQ ID NO:29) and 5'-GCAAAACATAAGGGGTGTCAACTTTACTTGA-3' (SEQ ID NO:30). The primer 5'-GACACCCCTTATGTTTTGCTGGC-3' (SEQ ID NO:31) and a primer annealing downstream of the region coding for the H transmembrane domain were used to amplify the MV H transmembrane encoding region. In the fusion step, the isolated PCR fragments with an overlapping sequence of 19 nucleotides (underline) were mixed and amplified with the external primers. The resulting fragment was digested with ClaI and EcoRI and then subcloned into peH5.--

Please replace the paragraph at page 52, line 4 with the following amended paragraph:

213
--Measles virus F protein was modified by changing the arginine position 109 and the lysine at position 111 to asparagine. Cloning of the viral glycoprotein (H and F protein) genes into the expression vector pCG under the control of the CMV early promoter has been described by Cathomen et al., 1995, supra. The F cleavage mutant (pCG-Fcm) with substitutions in the furin recognition motif was prepared by introduction of site-specific mutations (underlined) with the complementary primers Fcm1 (5'-GCTTCAAGTAGGAACCAACAGATTTGCGGG-3') (SEQ ID NO:32) and Fcm2 (5'-CCCGCAAATCTGTTGTGGTTCCTACTTGAAGC-3') (SEQ ID NO:33) into the double-stranded pCG-F plasmid using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). After dideoxy sequencing of the complete F gene, the mutagenized plasmid was used for transfection of 293 and Vero cells.--

Please replace the paragraph at page 58, line 8 with the following amended paragraph:

--In this example, the standard MV F cleavage signal RHKR (SEQ ID NO:15), recognised by furin, is mutated using random primers to generate a library with potentially novel cleavage specificities. To avoid restoring furin sensitivity, primers are designed such that positions 1,2,3, cannot be arginine or lysine.

214
Furin Cleavage ▼
1 2 3 4 5 6 7
MV F0 R H K R F A G (SEQ ID NO:34)
Furin sensitive site Beginning of F1 polypeptide
▲

Arginine is coded by (single amino acid codes):

AGG

AGA

CGG

CGA

CGC

CGT

Lysine is coded by:

AAG

AAA--

Please replace the paragraph at page 58, line 27 with the following amended paragraph:

--The possible permutations of bases (as a result possible amino acids) are as shown:

Furin cleavage ▼

1	2	3	4	5	6	7
R	H	K	R	F	A	G
				L	I	A

(SEQ ID NO:35)

Primer:

XAT XAT XAT XXX TTC GCA GGT	} (SEQ ID NO:36)--
XCC XCC XCC XXX TTA ATA GCT	
XTX XTX XTX XXX	

Please replace the paragraph at page 60, line 4 with the following amended paragraph:

--In one embodiment of the invention, the targeting specificity of an attenuated virus was altered by displaying a single chain antibody (ScFv) on the surface of the virus. In this embodiment, cDNAs coding for scFvs against a human lymphocyte differentiation antigens CD38 and CD52 were ligated into a full length infectious clone of MV-Edm (Figure 5). The genes were inserted as in-frame fusions linked to the C-terminal codon of the H glycoprotein through a linker sequence encoding an IEGR (single amino acid code) (SEQ ID NO:37) factor Xa protease cleavage signal. MV-Edm recombinant viruses were recovered from these constructs and were amplified in CD46 receptor-positive Vero cells in which they replicated as efficiently as unmodified MV-Edm. Correct expression of the scFV domains was confirmed on immunoblots of cell lysates of infected Vero cells, and probed with an antibody against the H glycoprotein (Figures 6A-D).--

Please replace the paragraph at page 63, line 4 with the following amended paragraph:

217
--CDNAs encoding the three forms of the scAb were transferred to a pCH-H vector (17) containing a Factor Xa cleavage site 3' to the H ORF from retroviral expression vectors (J. Zhang, data not shown) using PCR amplification (primer sequences: 5'-GCGCGCTGGCCCAGGTG-3' (SEQ ID NO:38) and 5'-TGCGGCCGCGCGTTTC-3' (SEQ ID NO:39), BssHII and NotI sites underlined). For detection purposes, an amino-terminal Flag tag (DYKDDDDK) (SEQ ID NO:40) was inserted downstream of the ATG start codon of each H construct. DNA sequencing confirmed the integrity of all constructs. The cDNA encoding HXL was transferred from the pCG construct into a molecular clone of MV-Edmonston, p(+)MV-Nse(18). Virus was rescued as previously described (19).--

Please replace the paragraph at page 70, line 15 with the following amended paragraph:

218
--The parental plasmids pCG-F and pCG-H code for the F and H proteins of MV-Edmonston (9). Plasmids pCG-H/SfiI/NotI and pCG-H/XsfiI/NotI, the second including a factor Xa protease (FXa) cleavage signal before the SfiI/NotI cloning sites, were constructed and digested with SfiI and NotI to provide the backbone in which the coding regions for the displayed domains were inserted. The constructs pCG-H/hEGF, pCG-H/XhEGF, pCG-H/hIGF1 and pCG-H/XhIGF1 were made by transferring the SfiI/NotI hEGF and hIGF1 fragments from pEGF-GS1A1 (Peng, k.W., 1997, Thesis. University of Cambridge)) and pIGFNA1 (Chadwick, et al., J. Mol. Biol. 285:485-494), respectively, into SfiI/NotI-digested pCG-H/SfiI/NotI and pCG-H/XsfiI/NotI. The coding sequence of the linker region (IEGRAAQPAMA, one letter code) (SEQ ID NO:41) is 5'-ATCGAGGGAAGGGCGGCCAGCCGGCCATGGCC-3' (SEQ ID NO:42). The four constructs were tested to verify their functionality in cell fusion assays.--

Please replace the paragraph at page 70, line 26 with the following amended paragraph:

219
--The PacI-SpeI fragments containing the hybrid H genes were corrected to comply to the rule of six (7/Calain, et al., J. Virol. 67:4822-4830, 1993)) by a PCR deleting one nucleotide between the stop codon (underlined) and the SpeI site (*italics*), the final sequence being 5'-TAGTAACTAGT (SEQ ID NO:43). The fragments were then inserted into PacI-SpeI digested p(+)MV-Nse (Singh, et al., J. Virol. 73:4823-4828, 1999) encoding the MV Edmonston

219
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antigenome, yielding plasmids p(+)MV-H/hEGF, p(+)MV-H/XhEGF, p(+)MV-H/hIGF1 and p(+)MV-H/XhIGF1.--

Please replace the paragraph at page 71, line 19 with the following amended paragraph:

220
--To introduce the SfiI/NotI cloning site at the C-terminus of the H sequence, thus enabling ligands to be inserted as SfiI/NotI fragments, oligonucleotides HXmabak (5'-CCG GGA AGA TGG AAC CAA TGC GGC CCA GCC GGC CTC AGG TTC AGC GGC CGC ATA GTA GA-3') (SEQ ID NO:44) and Hspefor (5'-CTA GTC TAC TAT GCG GCC GCT GAA CCT GAG GCC GGC TGG GCC GCA TTG GTT CCA TCT TC-3') (SEQ ID NO:45) were synthesised. When annealed, these two oligonucleotides form a DNA fragment with XmaI and SpeI cohesive ends which was ligated with XmaI/SpeI digested (pCG-H(SfiI-) backbone. The correct sequence of this construct, pCG-H-SfiI/NotI was verified by DNA sequencing.--

Please replace the paragraph at page 71, line 27 with the following paragraph:

221
--To make the construct pCG-H-FX-SfiI/NotI, which includes a factor Xa protease (FX) cleavage signal before the SfiI/NotI cloning sites at the C-terminus of the MV H sequence, oligonucleotides HXmaFXbak (5'-CCG GGA AGA TGG AAC CAA TAT CGA GGG AAG GGC GGC CCA GCC GGC CTC AGG TTC AGC-3') (SEQ ID NO:46) and HNotFXfor (5'-GGC CGC TGA ACC TGA GGC CGG CTG GGC CGC CCT TCC CTC GAT ATT GGT TCC ATC TTC-3') (SEQ ID NO:47) were synthesised. When annealed, these two oligonucleotides form a DNA fragment with XmaI and NotI cohesive ends which was ligated with the XmaI/NotI digested pCG-H-SfiI/NotI backbone. The correct sequence of this construct was verified by DNA sequencing.--

Please replace the paragraph at page 75, line 1 with the following amended paragraph:

222
--Plates were washed three times with 200 µl TBS and bound virus was detected by incubation with 100 µl of a rabbit anti-Hcterm specific antiserum diluted 1:100 in 1% blocking solution for 2 hours at 4°C . The Hcterm antiserum was raised in rabbits against a peptide corresponding to the 12 H-protein carboxy-terminal amino acids (NH2-CTVTREDGTNRR) (SEQ ID NO:48) linked to keyhole limpet hemocyanin through the naturally occurring cysteine

A23
Circled
(C). For detection, peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno Research, 111-035-003) diluted 1:5000 in 1% blocking solution was added for 1 hour at 4°C and after intense washing the color reaction was performed using the POD substrate from Boehringer Mannheim (1 363 727).--

Please replace the paragraph at page 76, line 3 with the following amended paragraph:

A23
--The human epidermal growth factor (hEGF, 53 amino acids) were used to generate a hybrid protein in which the hEGF coding region was fused in frame with the H coding region, but eliminating the last two arginine residues, to avoid the possibility of introducing an undesired furin cleavage site (H/hEGF, Fig. 14A, second line from bottom). A flexible linker region (AAQPAMA) (SEQ ID NO:49) was added between the domains to increase the probability of independent folding function. In another embodiment, the human insulin-like growth factor-1 (hIGF1, 70 amino acids) was fused to the H protein. A factor Xa protease cleavage site (IEGR; SEQ ID NO:37) was added before the linker region (H/XhEGF, Fig. 14A, second line from bottom). Hybrid H/hIGF1 and H/XhIGF1 proteins were constructed (Fig. 14A, bottom line). When the hybrid proteins were co-expressed with a MV F protein, H/hEGF and H/XhEGF retained the same level of fusogenicity as parental H, whereas fusogenicity of H/hIGF1 and H/XhIGF1 was reduced but remained clearly over background.--

In the claims:

Please cancel claims 1-26 without prejudice.

Please add claims 27-58 as follows:

A24
--27. A method of monitoring gene expression from virus infected cells within an organism, said method comprising:

(a) administering a Paramyxoviridae virus to said organism, wherein said Paramyxoviridae virus comprises a nucleic acid sequence encoding a heterologous polypeptide, and wherein said heterologous polypeptide is released from infected cells into a biological fluid of said organism when expressed, and